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A CYTOLOGICAL STUDY OF THE STAMENS OF SMILAX HERBACEA*

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INTRODUCTION.

There seems to be a general agreement among the various investigators of the reduction division, that there is a pairing and conjugating, in the first reduction division, of the univalent chromosomes to form bivalents, but there is a considerable diversity of opinion as to the time of the pairing and fusion. Allen, Gregoire, Overton and many others hold the view that there is a side to side pairing of the chromatic elements occurring usually about the time of "synapsis." De Vries also claimed that there is a side to side pairing, but was not certain when it occurred, although it was some time before the separation of the halves of the bivalent chromosomes. As a proof of this theory it was held that, since a longitudinal split of the spirem is discernible in the early stages of the reduction division, the double spirem was the result of a conjugation of two simple spirems. But according to Schaffner, Farmer and Moore, Mottier and others the early split is a longitudinal division of the same nature as that which occurs at each vegetative karyokinesis. The pairing of the univalents according to this view must occur very early, before the formation of the spirem; and the protochromosomes, which in some species are rather definite masses and approximate the reduction number of chromosomes, probably represent the end of the stage when the pairing occurs.

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In my studies, therefore, careful observations of the spirem were made with the view of determining whether there is a continuous thread or whether there are a number of short individual threads interwoven but distinct as described by Lawson and others in a number of cytological studies of plants more or less closely related to S. herbacea.

The exact manner of chromosome formation was also studied to determine whether they were the result of a looping and a later longitudinal folding, or if there was simply a transverse constricting and breaking apart of the spirem to form the chromosomes as described by Miss Elkins in Smilax herbacea.

It was with these points in view that this study and review of the necessary literature was taken up under the guidance of Prof. John H. Schaffner, whose assistance and advice was found to be of inestimable help in all work undertaken with him.

GENERAL CONSIDERATION OF PAST LITERATURE ON THE SUBJECT.

Since in recent years all except the latest papers have been repeatedly reviewed, it is not considered necessary to refer to any except such as have a very direct bearing on the matter in hand. Those dealing with plants closely related to Smilax herbacea are however included so far as they are available for study.

Miss Elkins in her paper, "The Maturation Phases in Smilax herbacea," states that she did not find a distinct reticulum in the microsporocytes, and often the chromatin bodies were in pairs or fours scattered through the finely granular meshes. According to her account the multinucleolate condition is the rule rather than the exception and often the nucleoli have papillate projections which are present quite late. At "synapsis" or contraction there is never more than one nucleolus present which condition is brought about by the union of the nucleolar elements, but often there are dark staining bodies left in the nuclear cavity. She also found that the nucleolus disappears at the metaphase just as Gates found for Oenothera rubrinervis. In the presynaptic stages, the linin meshes are said to contract, drawing the chromatin material together, while the nucleolus is at one side projecting from a mass of threads. It is during this period that she found the chromatin becoming arranged into an interwoven beaded filament. The appearance of the nucleus after synizesis is stated to be quite different from its previous condition, the chromatin emerging as a homogeneous filament. It is also vaguely suggested that this may facilitate proper placing of the paired parental elements in the chromosomes in the spirem. She says that the chromosomes do not appear as definitely united until the segmentation of the spirem. After synizesis the spirem is a fairly thick thread, slightly beaded, but in a short time becomes homogeneous. She observed that the double character of the

spirem was discernible at this time and at intervals the spirem, which is made up of previously paired chromatin elements, was constricted in some places to a narrow thread and finally separated into irregularly shaped double segments. These pieces continue to thicken and shorten, forming X and V-shaped chromosomes. She says that the first division is merely a separation of the chromosomes, but the second is a true mitosis. At the telophase, she observed that the spirem was disposed about the periphery of the newly formed membrane. The nuclear membrane disappears and the spirem is spread out over the spindle and in a short time the spirem contracts into the equatorial plane, dividing into chromosomes which become attached to the spindle with the open ends outward. She could not determine the exact number of chromosomes, but decided that there were either twelve or thirteen.

Schaffner found in his study of Erythronium that the spirem was at first long, slender, with chromatin granules that are not prominent before the looping. The spirem undergoes a contraction and a preceptible thickening, and is thrown into twelve loops which are apparently broken apart by the twisting and contracting. The chromosomes are said to be of various sizes and seem to be double. They are attached to the spindle near the free ends and during metakinesis are uncoiled and pulled apart in the middle.

In Lilium tigrinum, (13), he found the chromatin network forming a thin spirem with a single row of spherical granules. There were no free ends so this would point to the fact that the spirem is continuous and is also free in the cavity. The spirem was then found to be in a condition of contraction and there was not any apparent change in the spirem after it had come out of this condition. After this the linin thread is said to elongate. The spirem also has a tendency to form into loops. Twelve loops are formed which break up into twelve chromosomes. These are attached to the spindle fibers near the free ends in the mother star and are separated by a transverse division. The split in the second division is a longitudinal one.

When working with Agave virginica (15), he found that there was a course chromatin net present and the cytoplasm was dense and spongy. The chromatin net stretched out and formed bivalent protochromosomes which in turn formed a delicate spirem with a single row of granules. Synizesis followed, and in a study of the living material no contraction of the chromatin material was noticeable. After synizesis a transverse division of the chromatin granules takes place with a shortening and thickening of the spirem which is thrown into loops of various sizes and pressed against the wall of the nuclear cavity. With the breaking of the spirem there results three ring chromosomes,

five smaller, and four large, long ones, which are rather well individualized. The chromosomes are attached to a bipolar spindle and are said to undergo a transverse splitting or breaking at the loop end in the first division and a longitudinal separation occurs in the second.

Lawson made a study of the microspores of several plants and arrived at a number of new conclusions in regard to the relation of "Osmosis as a Factor in Mitosis," (5). He said that the nuclear membrane did not break down or disappear during the development of the spindle, but acted as any permeable membrane would under varying osmotic conditions. He gave drawings showing that when the amount of nuclear sap became very much reduced, the membrane drew close to each chromosome and finally there were as many osmotic systems as there were chromosomes and each chromosome has its own sphere of "kinoplasm." He holds that the achromatic spindle is simply an expression of the tension of the cytoplasm and is not an active factor in mitosis.

In his paper, "The Phase of the Nucleus known as Synapsis," (4), he states that the condition described is not a contraction at all and has nothing to do with the fusion of maternal and paternal chromatin, so was not a critical stage in reduction. In his study of Smilacina he did not find protochromosomes, but the reticulum was found to be made up of a number of linin threads which approximate the diploid number of chromosomes. Since he found no vacuoles in the cytoplasm he concluded that the nuclear cavity itself was acting as a vacuole, since the sporocytes were still enlarging and also on account of the turgid appearance of the nucleus. By the stretching of the nuclear membrane, the space within was increased causing a great osmotic pressure, which he concluded facilitated growth. This condition is probably synonymous with that described by many authors as "synaptic contraction." By actual measurements he stated that he was able to determine that there was no contraction whatever. Thus the conclusion reached in the paper was, that "synapsis" is simply a period of growth during which the great amount of nuclear sap causes the nuclear membrane to distend and withdraw from the chromatin material. This was all explained as occurring before reduction division, because all the sporocytes had merismatic activity which manifests itself in the two divisions immediately following.

Schaffner in his paper, "Synapsis and Synizesis" (14), defines synapsis as the formation of bivalent chromosomes from univalent ones by an end to end fusion and a subsequent folding. McClung's term Synizesis was accepted as appropriate for the contractions usually observed in prepared sections showing early stages in reduction. Synizesis was explained as an artifact probably due to plasmolysis.

Sauer, when investigating Convallaria majalis (10), found that there was a resting period after the last archesporial division, but that in a short time a chromatin network was formed. The nucleous described as being visible from the beginning, fragments in the later stages forming several micronucleoli. He says that there is a clear area in the nucleus and that the continuity of the spirem is very evident. After synizesis a loosening and unwinding of the thread begins. The linin thread becomes thicker and the chromatin granules elongate. Altho the spirem is shorter it occupies the whole cavity and the division of the granules is apparent. After this stage the doubleness of the spirem is no longer visible. The spirem is next thrown into sixteen loops which later divide into sixteen chromosomes. The first division of the chromosomes in the microsporocytes is transverse and therefore qualitative.

Miss Hyde found in Hyacinthus orientalis (3), a definite network in the microsporocyte, but fails to discover any accumulation of chromatin material that might be interpreted as protochromosomes. She determined, however, that the complicated spirem was continuous, undergoing synizesis, looping, and finally breaking into eight well individualized chromosomes.

Miss McAvoy, in her observations of the reduction division in Fucshia (7), found protochromosomes which seemed to stretch out and form a continuous spirem with chromatin granules. The spirem undergoes synizesis after which the delicate thread soon begins to thicken and in a short time shows loops which lie along the periphery of the nucleus. These loops, fourteen in number, break apart to form fourteen chromosomes.

The study that she made of Oenothera biennis (8), served to confirm the results stated in her previous paper in as much as she found a reticulum and protochromosomes which in turn formed a continuous spirem that could be traced its entire length. The synizetic knot is not so tight as in some plants and even in this stage she was able to trace out much of the spirem. Loops were formed which break apart forming seven chromosomes.

MATERIALS AND METHODS.

The primary purpose of this study was to observe the reduction division in the microsporocytes of Smilax herbacea and also to incidentally consider any peculiarities in relation to the degeneration of normal stamens to vestigal structures or to their complete disappearance. It was found, however, that the material available did not give the more critical stages bearing upon the second part of the problem.

The material used in the investigation was collected from the first week in May, 1913, at Columbus, Ohio, to the middle of June, 1913, near the Lake Laboratory at Cedar Point. The

buds were killed in Schaffner's weaker chrom-acetic acid with a trace of osmic acid added, being left in this for twenty-four hours. After being thoroughly washed in water, the material was dehydrated by passing it through the various grades of alcohol to 70%, where it was left until September, when it was passed through the higher grades into chloroform, from which it was gradually passed into pure parafine and imbedded. Sections 10μ to 13μ thick were cut.

Several methods of staining were used. The first tried was analin safranin, which was a fairly good stain, but it did not make enough differention between the chromatin material and the cytoplasm to be easily studied. Next Heidenhain's iron-alum haemotoxylin was used and found to be very good, staining the chromatin material black and the surrounding tissues brownish. In using this stain, the slides were passed through turpentine, xylol, the different grades of alcohol to water, then passed into iron-alum, where they were left for two hours; after being well washed in water they were left four hours or longer in Heidenhain's haemotoxylin after which they were washed and placed in iron-alum to clear, and after dehydrated they were mounted in Canada balsam. The most satisfactory stain was Delafield's Haemotoxylin. The slides were passed through the alcohols to 25%, then into Delafield's Haemotoxylin where they were left for two hours, after which they were washed in water and passed up through the alcohols and mounted.

INVESTIGATION.

The earliest preparations show the resting cells after the last archesporial division, but before the tapetum has become differentiated. In the youngest sporocytes the nuclei are small, measuring 9μ or 10μ, and the cells fit closely together forming a compact mass. In many nuclei there are several nucleoli present which do not appear spherical, but have one or more finger-like projections. In the youngest sporocytes the chromatin material seems to be arranged in a loose reticulum (Fig. 1), which is not uniformly spaced throughout the nuclear cavity, and is easily distinguished in it. Following this reticular stage the chromatin material has a tendency to draw together in masses which are rather definite in shape, spongy and flaky in appearance, and have fine threads radiating in all directions from the central lumps. (Fig. 2).

There is a tendency for these spongy masses to become more compact and definite in shape, approximating the reduced number of chromosomes, (Fig. 3), and without doubt these are the protochromosomes described by various authors, and designated as "prochromosomes" by Overton and Strasburger. It is probably at this stage that the univalent chromosomes are paired in

order that they may have a definite position in the spirem during the synaptic stages when the bivalent chromosomes are formed by an end to end pairing and later longitudinal folding of the chromatic elements. By many investigators "synapsis" is used to designate the period of contraction which very generally appears in the earlier stages of reduction. But it is much better to use the term synizesis as was suggested by McClung and adopted by Schaffner in the more recent of his cytological papers. By eliminating this confusion of terms such expressions as "synaptic mates," etc. in relation to the chromosomes, become intelligible without further explanations.

The protochromosomes do not retain a definite shape, but in a short time there is an apparent elongation of each mass and a tendency for the delicate connecting linin threads to become thicker as the elongation continues. (Figs. 4, 5, 6). Soon no traces of the flaky masses are left, but instead there is a very delicate continuous spirem which can be traced for long distances in many of the sporocytes without finding any free ends. The free ends in most cases can all be accounted for by their having been cut in sectioning. (Fig. 7).

There is now a perceptible enlargement of the nucleus, which appears very turgid; as a result of this enlargement the very delicate spirem becomes loosened from the nuclear membrane and does not appear to be so uniformly arranged about the periphery as before, but has the appearance as if it had been treated with some plasmolizing reagent. (Fig. 8).

By this time there is usually one large nucleolus present, which very seldom appears in a central position and sometimes there are also dark staining granules in the nuclear cavity which in all probability are minute nucleoli. Miss Elkins noted this same fact in her study of Smilax herbacea.

The spirem and granules in the earlier division stages show no evidence of a double character. Soon after the spirem has become loosened from the nuclear wall, there is an irregular massing of the thread, which either may or may not enclose the nucleolus. (Figs. 9, 10, 11). The types of contraction are not always the same and there was no evidence that synizesis is an actual stage in the reduction division. As previously mentioned Lawson considered this condition to be due to a period of growth in the nucleus, there would be thus no actual shrinking of the chromatin, but there can be no question that in the preparations studied there was a considerable actual contraction. Schaffner (15) regarded this condition as an artifact on account of experiments tried with living material of Agave virginica and the reactions also obtained by the treatment with different reagents which caused plasmolysis to take place in the vegetative cells, giving the spirem much the same appearance that was found in

many reduction preparations of apparently the same age. Miss Elkins regards this "synapsis" as a natural stage in the reduction division and not as an artifact as the many types of synizetic masses lead the writer to believe. The synizetic knot is not necessarily found to one side of the nuclear cavity, but is often in the center in which case the nucleolus is usually found to be lateral in position. Often threads with numerous granules are seen projecting from the greater mass of chromatin material toward the periphery of the nucleus. Before the contraction of the spirem, there were no double granules observed and the spirem was single, but following synizesis a heavy spirem extends throughout the nuclear cavity touching the periphery at various points. (Figs 12, 13, 14). No evidence whatever in favor of the theory that the double spirem is the result of the conjugation of two simple spirems was found. The evidence rather points to a longitudinal splitting instead of a conjugation. (Fig. 14).

The heavy spirem which often showed very plainly its double character is thrown into loops around the periphery of the nuclear cavity and in an older sporocyte each incipient loop appeared to have twisted more tightly together, showing as definite bodies still connected together so that almost the entire length of the spirem may be traced by following the twists of the loops. Miss Elkins described the chromosomes as being formed by the halves of the double spirems constricting at intervals until only a very slender thread united the segments, but the writer found a number of preparations which showed well defined loops in which the twisted condition appeared plainly just at the time when they were pulling apart, as seen in Figure 17. Often large granules are seen upon the linin thread even after well twisted loops are formed and the double character of the thread is seen even in the fully formed chromosome, if one focuses carefully.

By the transverse pulling apart of the heavy looped spirem, there results rather indefinitely shaped chromosomes which are joined together for some time by very delicate threads. (Figs. 18, 19, 20, 24). The irregular masses tend to shorten and thicken forming twelve rather well individualized chromosomes. (Figs. 21, 22, 23). In many of the preparations of this stage it is impossible to count the chromosomes because of their proximity and the irregularity of shape.

After the chromosomes have acquired their individual shape they are still connected by fine threads (Fig. 24) and the nuclear membrane becomes indistinct while the incipient spindle appears about it. (Figs. 24, 25). The membrane disappears and a definite bipolar spindle is apparent from the beginning with the chromosomes and their connecting threads arranged over it. The chromosomes appear to be gradually pulled into an equatorial position by a shortening of the connecting threads. During this

change the nucleous disappears. It is not possible to discover whether it was dissolved or disintegrated into smaller bodies and ejected into the cytoplasm. The cytoplasm at this stage has a very spongy appearance, but no micronucleoli were seen in it.

In the mother star of the first division the chromosomes are attached to the spindle fibers near their free ends with the head of the loop extending outward as found by Schaffner in Lilium philadelphicum (11) and by Miss Hyde in Hyacinthus (3). There is a gradual shortening of the spindle fibers and at the same time the chromosomes uncoil and pull apart at the outer head of the loop or at the point where fusion took place during synapsis. From drawings of metakinesis it will be seen that the transverse splitting of the chromosomes of Smilax herbacea is not simultaneous as is found in many plants. (Fig. 27). After metakinesis the chromosomes are arranged around the poles forming the daughter stars of the first division. (Fig. 28). There is also a perceptible increase in the density of the cytoplasm in the equatorial region where in a short time a distinct cell plate is seen. By the time of the complete formation of the cell plate, the spindle is no longer visible and a new nuclear membrane is laid down around the daughter masses of chromatin material thus forming two new cells very similar to the parent cell, but much smaller. With the formation of the new nuclear membrane, it is also found that the nucleoli of the daughter cells are beginning to appear. The chromatin material in these daughter cells does not undergo such changes as were evident in the nuclei of the sporocyte, but the newly formed chromosomes are massed together not to form a continuous spirem, but an irregularly shaped mass in which the individual chromosomes may be distinguished. (Fig. 29).

The daughter cells do not immediately separate, but may be seen still clinging together after the second division is well advanced. In the second division the chromosomes are attached to the spindle fibers in the equatorial plane by the head of the chromosome, having the free ends extending outward. (Fig. 30). The separation of the chromosomes at this division is along the longitudinal split. After the second metakinesis we find the two daughter stars with the distinct chromosomes (Fig. 31) which were readily counted in several preparations from the polar views. The number was found to be twelve. (Fig. 32).

The cell plates of this division soon appear and a new nuclear membrane is evident in each daughter cell around the rather small chromosomes which become more or less crowded together and connected by fine connecting strands. All the tetrads appeared to be normal, there being no such irregularities found as shown by Fullmer in Hemerocallis and by Miss McAvoy in Fuchia.

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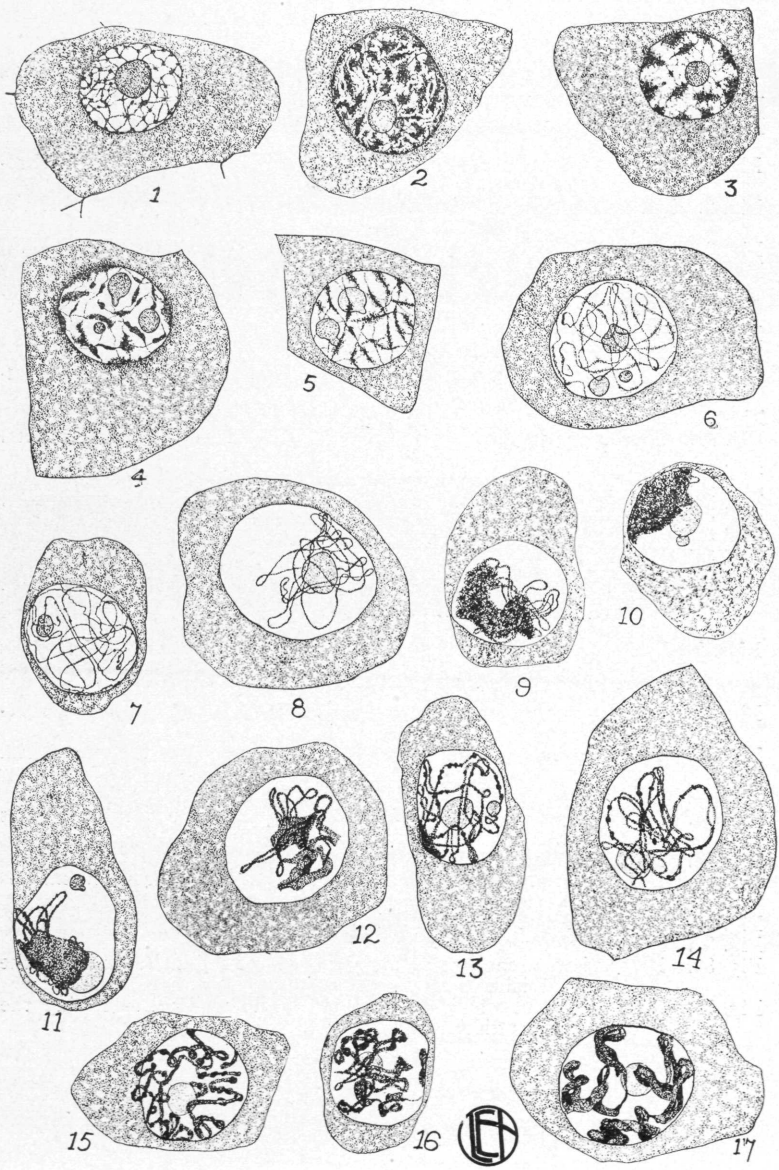
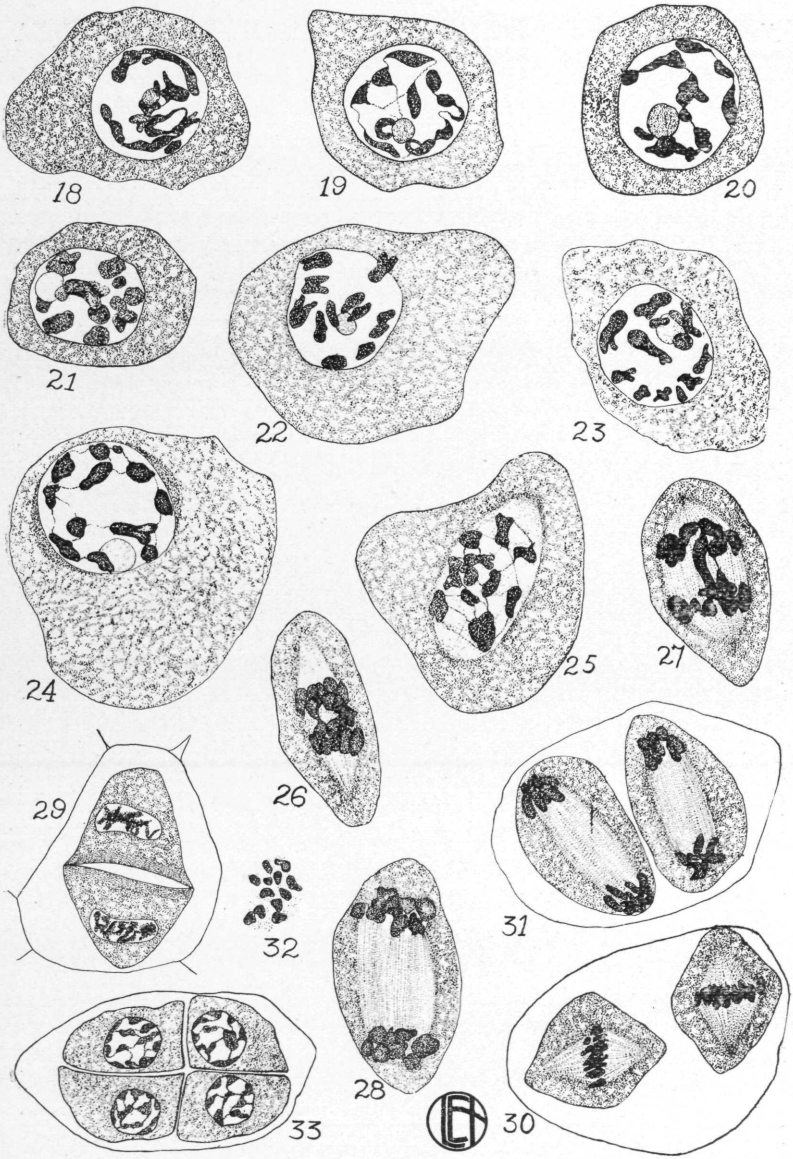
Explanation of Plates XVI and XVII.

The plates were reduced $\frac{5}{8}$ in reproduction. All the drawings were made with a compensating ocular 12 and a 1-16 oil immersion lens. An Abbe camera lucida was used.

Fig. 1. Microsporocyte before the beginning of the division of the chromatin network.
Fig. 2. Microsporocyte showing the flaky and spongy appearance of the chromatin material.
Fig. 3. Masses of chromatin material which are the protochromosomes.
Figs 4, 5. Later stages showing the elongation of the protochromosomes in their tendency to form a spirem by stretching out along the linin thread.
Fig. 6. Early spirem with irregular flakes along its sides.
Fig. 7. Early spirem with small granules.
Fig. 8. Microsporocytes showing the spirem free from the nuclear membrane and collapsing.
Figs. 9, 10. Sporocytes showing different types of synizesis.
Fig. 11. Sporocyte in synizesis with the projecting strands showing granules.
Fig. 12. A synizetic knot with rather heavy projecting loops.
Fig. 13. Heavy spirem showing granules and beginning of looping.
Fig. 14. Sporocyte showing the double nature of the spirem and granules.
Fig. 15. Sporocyte showing the early looping stage and double spirem.
Fig. 16. Sporocyte showing well formed loops.
Fig. 17. Chromatin loops completely formed and just breaking apart.
Figs. 18, 19, 20. Sporocytes showing the prominent chromosomes that have not completely separated, but still show some connecting threads.
Figs. 21, 22, 23. Sporocytes showing the twelve mature chromosomes; the looped nature of the chromosomes is still evident in most cases.
Fig. 24. Sporocyte showing the delicate connections between the chromosomes and the incipient spindle.
Fig. 25. Chromosomes in the spindle being drawn into the equatorial plane.
Fig. 26. Early stage of metakinesis showing the chromosomes dividing.
Fig. 27. Later stage of metakinesis showing most of the chromosomes divided.
Fig. 28. Daughter star of the first division.
Fig. 29. Daughter cells showing the more or less distinct chromatin masses in the nuclei.
Fig. 30. Mother star of the second division.
Fig. 31. Daughter star of the second division.
Fig. 32. Polar view of the twelve chromosomes of a daughter star of the second division.
Fig. 33. Normal tetrad within the old sporocyte wall still showing the more or less distinct daughter chromosomes.



Humphrey on "Stamens of Smilax."



Humphrey on "Stamens of Smilax."